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Differential expression of versican isoforms is a component of the human melanoma cell differentiation process☆

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Abstract

Versican is a large chondroitin sulfate proteoglycan produced by human melanoma cell lines and malignant melanocytic lesions. In the present work, we have analyzed the expression of versican spliced variants V0, V1, V2 and V3 in human melanoma cell lines at several differentiation degrees. The isoform expression pattern depends on the degree of cell differentiation. Differentiated cell lines do not produce any of the versican isoforms as analyzed by Western blot, Northern blot and RT-PCR. All cell lines with an early or intermediate degree of differentiation (AX3, SK-mel-37, Rider, SK-mel-1.36-1–5 and SK-mel-3.44) expressed V0 and V1 transcripts, whereas V2 and V3 expression was shown only by the undifferentiated cell lines SK-mel-1.36-1–5 and Rider. Furthermore, we have analyzed the expression of versican isoforms in SK-mel-3.44 and SK-mel-1.36-1–5 cells induced to differentiate by TPA treatment. The expression of the large V0, V1 and V2 isoforms practically disappears in differentiated cells, whereas V3 remains detectable by RT-PCR analysis.

Keywords: Versican; Melanoma; Cell differentiation; Proteoglycan

1. Introduction

Melanoma cells can vary widely in their degree of pigmentation, cell morphology and growth rate, and these differences can be observed in different metastases and even among the various cells within individual lesions of melanoma patients. It has been suggested that melanoma heterogeneity reflects a corresponding diversity in the phenotype of normal cells undergoing melanocyte differentiation [1,2]. According to this view, patterns of gene expression change as cells progress through distinct stages in the melanocyte lineage.

The extracellular matrix (ECM) surrounding the cells is an important element controlling cell behavior in aspects such as cell proliferation, adhesion and migration. One of the main ECM components are proteoglycans (PGs). Altered PGs have been described in tumors and transformed cells, and are believed to contribute to the abnormal assembly of the ECM in those cells [3–6]. Tumor cell ECM is usually abundant in hyaluronan, which forms a tridimensional network with chondroitin sulfate proteoglycans from the hyalectan family as well as with other molecules [7–9].

We have previously described the presence of versican, a member of the hyalectan family in human melanoma cell lines and malignant melanoma in vivo and related the role of versican to the biological properties of melanoma cells [10]. In that work, we have shown that versican expression clearly correlates with melanoma cell differentiation stage since only cell lines with an early and intermediate differentiation degree, but not differentiated cells, produce versican.

Versican, as well as the other members of the hyalectan family, displays three structural domains: the N-terminal region (G1 domain) consists of an immunoglobulin-like loop and two link protein-like tandem repeats and is responsible for hyaluronate binding; the central domain carries the glycosaminoglycan side chains and the C-terminal globular region (G3 domain) consists of two EGF-like elements, a C-type lectin domain and a CRP-like (sushi) module; it may interact with simple carbohydrates and glycosaminoglycans and probably with other proteins such as tenascin-R [11,12]. The central versican domain consists of two large subdomains, designated GAG-α and GAG-β, which are encoded by two alternatively spliced exons. In mammals, versican appears as four possible spliced variants: V0 is the largest one and contains both GAG-α and GAG-β;

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the other variants are V1 (contains GAG-β), V2 (contains GAG-α) and V3 (lacks any GAG subdomain).

In the present work, we have further analyzed the expression of spliced versican isoforms in human melanoma cell lines with different degrees of differentiation as well as investigated the relationship between versican expression and the melanoma cell differentiation process induced by 12-*O*-tetradecanoyl-phorbol-13-myristate acetate (TPA).

2. Materials and methods

2.1. Cell culture

Human melanoma cell lines SK-mel-131 (cl 1.36-1–5), SK-mel-131 (cl 3.44), SK-mel-23, SK-mel-37, Rider, AX3, MeWo and DX2 originally derived from human melanomas by Houghton et al. [2] were obtained from Dr. F.X. Real (IMIM-IMAS, Barcelona, Spain). Cells were grown in a humidified atmosphere at 37 °C with 5% CO_2 in DMEM medium supplemented with 10% fetal calf serum, 100 IU/ml penicillin and 100 μg/ml streptomycin (all from GibcoBRL/Life Technologies, Paisley, Scotland). The medium was then removed, a cocktail of protease inhibitors was added (10 mM EDTA, 5 mM benzamidine (Sigma, St. Louis, MO) and 1 mM PMSF (Boehringer Mannheim, Mannheim, Germany)) and analyzed by SDS-PAGE in a 6% polyacrylamide gel, as described by Laemmli [13]. To assure that the conditioned medium corresponding to the same number of cells was used, duplicate wells were seeded and cells were counted in a Neubauer chamber.

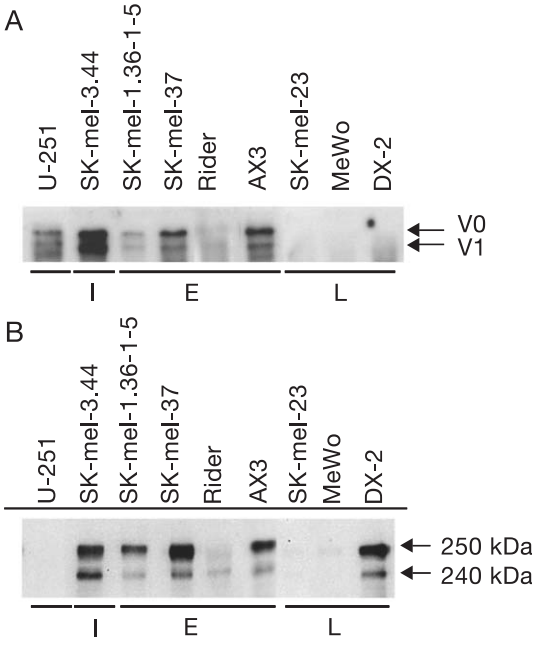

Fig. 1. Identification of versican isoforms and mel-CSPG in human melanoma cell lines. Conditioned media from human melanoma cell lines and U-251 astrocytoma cell line were treated with chondroitinase ABC and analyzed by Western blot with: (A) the antibody raised against versican and (B) the monoclonal antibody B5 raised against the melanoma-specific proteoglycan mel-CSPG as described in Materials and methods. I, intermediate differentiation degree; E, early differentiation degree; L, late differentiation degree. The 240 and 250 kDa bands corresponding to distinct glycosylated forms of mel-CSPG are shown.

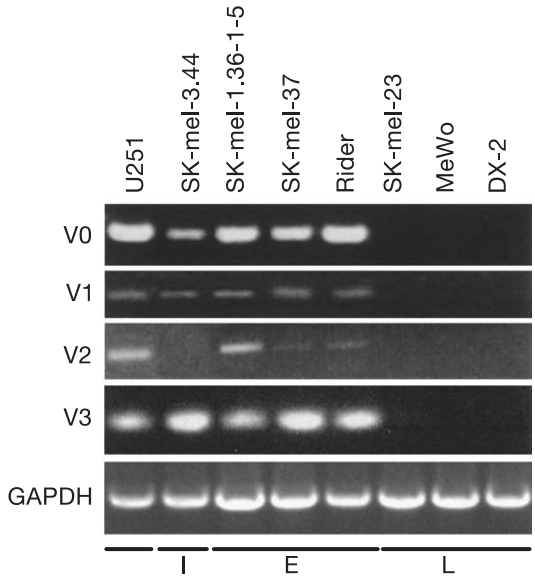

Fig. 2. Detection of versican isoforms (V0, V1, V2 and V3) in human melanoma cell lines using RT-PCR. RT-PCR was performed with total RNA from U-251 astrocytoma and melanoma cell lines with an early (E), intermediate (I) or late (L) differentiation degree as described in Materials and methods. Glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) served as a positive control.

For differentiation studies, 2×10^4 cells were seeded in 6-well plates in complete medium. After 24 h, medium was supplemented with 160 nM TPA (Sigma) dissolved in DMSO (Sigma) [1]. Control cells were grown in DMEM or DMEM supplemented with 0.0016% DMSO. Medium was changed every 72 h and kept at − 20 °C in the presence of protease inhibitors.

2.2. Western blot analysis

Enzymatic digestions were performed at 37 °C for 16 h with chondroitinase ABC (Sigma) (50 mU/ml in 33 mM sodium acetate, 33 mM Tris–HCl pH 8.0). The incubations were terminated by boiling the samples for 5 min. Samples were analyzed in a 6% polyacrylamide gel under reducing conditions. After electrophoresis, proteins were transferred onto Immobilon-P membranes (Millipore Corporation, Bedford, MA). The blot was placed in a blocking solution consisting of 5% skim milk in 0.05 M Tris Buffer, 0.15 M NaCl (TBS)-0.05% Tween-20 (Serva, Heidelberg, Germany) and incubated for 1 h at room temperature. The membranes were incubated with the polyclonal antibody against versican raised in our laboratory (1:1000) [10] or the monoclonal antibody B5 raised against mel-CSPG (1:50) (kindly provided by Dr. F.X. Real, IMIM-IMAS, Barcelona) [14] in 5%

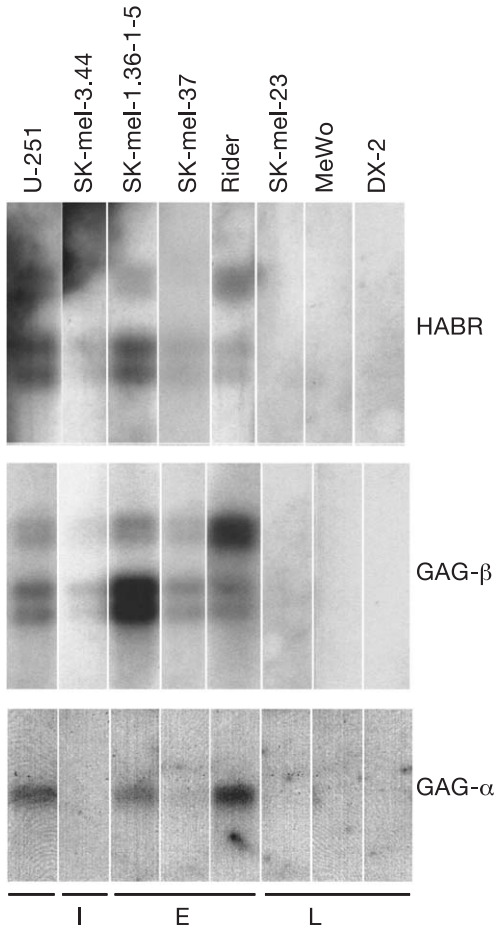

Fig. 3. Northern blot analysis of versican isoforms in total RNA from human melanoma cell lines. Versican isoforms mRNA was specifically detected with probes covering sequences coding for the hyaluronan-binding region (HABR), the glycosaminoglycan attachment subdomain GAG-β or the glycosaminoglycan attachment subdomain GAG-α.

skim milk in TBS-T for 16 h at 4 °C, washed and visualized by chemiluminescence (ECL System, Amersham Pharmacia Biotech, Buckinghamshire, England).

2.3. Immunocytochemistry

Cells were grown in cover slips, rinsed with PBS and fixed with 3% paraformaldehyde-2% sacarose for 20 min at room temperature. After rinsing with PBS, cells were permeabilized with 0.1% Triton X-100 in PBS for 10 min and nonspecific binding sites were blocked for 20 min with 1% BSA/0.02% goat serum. Cells were then incubated for 3 h at 37 °C with the anti-versican antibody (1:1000), the B5 antibody against mel-CSPG (1:50) or with the M111 antibody against gp110 (1:100) [1] (kindly provided by Dr. F.X. Real, IMIM-IMAS, Barcelona). Subsequently, cells were washed four times with PBS and incubated with the corresponding secondary antibody labeled with FITC (versican, M111) (Southern Biotechnology Association, Birmingham, AL) or TRITC (B5) (Sigma). Nuclei were visualized by incubating the cover slips with Hoetsch (0.1 mg/ml in PBS) for 3 min. Cultures were studied with a Nikon Eclipse E800 epifluorescence microscope and photographed with an integrated camera system.

2.4. RNA isolation, Northern blot hybridization and RT-PCR

Total RNA was extracted with RNeasy isolation kit (Qiagen, Hilden, Germany) from subconfluent cells. For Northern blot experiments, 15 μg of total RNA were separated by 1% agarose-formaldehyde gel electrophoresis and subsequently transferred onto Hybond-XL membranes (Amersham Pharmacia Biotech). The following [alpha-^{32}P]

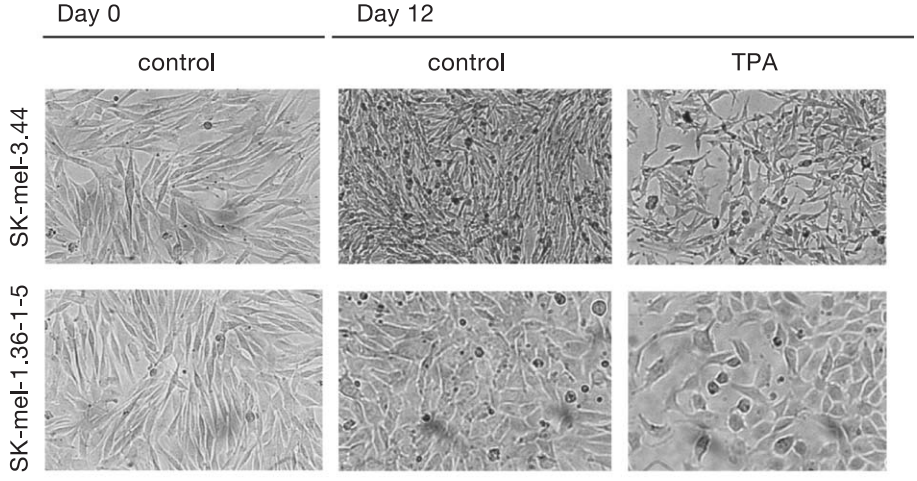

Fig. 4. Morphological changes in SK-mel-3.44 and SK-mel-1.36-1–5 human melanoma cell lines after induction of differentiation with TPA treatment. Cell cultures were treated with 160 nM TPA as described in Materials and methods. Cultures were viewed with a Leica Fluovert-Fu microscope and photographed with an integrated camera system.

dCTP-labeled probes were used: D7 (HABR, hyaluronan binding region), Genomic PCR (GAG-α) and C4 (GAG-β) as described by Dours-Zimmermann and Zimmermann [15].

For RT-PCR, 1 μg of total RNA was reverse transcribed in a 20 μl reaction volume with the Expand Reverse Transcriptase (Roche, Mannheim, Germany) as described in the protocol provided by the supplier. Ten microliters of the reaction mix were subsequently used for PCR. The primers used for amplification of the specific splice variants of versican were those described in Paulus et al. [16]. Thirty cycles were performed, each cycle including 1 min at 94 °C, 1 min at 55 °C for V0 and V1 or at 53 °C for V2 and V3 isoforms and 1 min at 72 °C. The final extension step at 72 °C was carried out for 10 min. $MgCl_2$ concentration was 2 mM for V1 and V2, and 2.5 mM for V0 and V3. The size of the amplification products were 351 bp (V0), 386 bp (V1), 373 bp (V2) and 342 bp (V3). As positive control, we used RT-PCR amplification of a 960-bp fragment of glyceraldehyde 3-phosphate dehydrogenase (for GAPDH primers, see Paulus et al.; amplification conditions: 30 cycles including 1 min at 94 °C, 1 min at 55 °C and 1 min at 72 °C; 1.2 mM $MgCl_2$). As a control for DNA contamination of the RNA preparation, PCR was performed without the initial reverse transcriptase step. Amplification products were resolved on 2% agarose gels stained with ethidium bromide. All the amplification fragments were isolated and digested with an appropriate restriction enzyme to confirm their identity (not shown).

3. Results

3.1. Identification of versican and mel-CSPG in human melanoma cell lines

Conditioned media from a number of human melanoma cell lines subconfluent cultures were collected, treated with

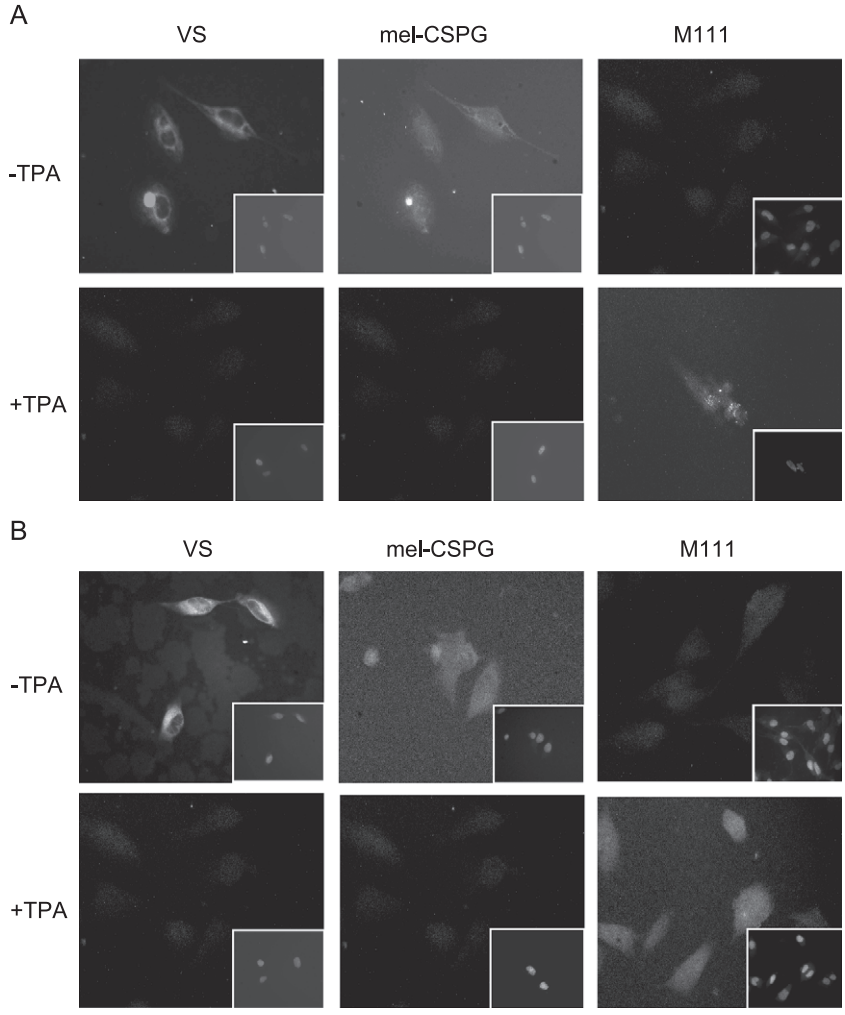

Fig. 5. Changes in the expression of versican and differentiation markers during the process of melanoma differentiation induced by TPA. Subconfluent SK-mel-3.44 (A) and SK-mel-1.36-1–5 (B) melanoma cells were grown in cover slips and treated with TPA to induce differentiation as described in Materials and methods. After 12 days of treatment, cells were fixed with paraformaldehyde. After permeabilization, cells were incubated with the anti-versican antibody, or with the monoclonal antibody B5 against mel-CSPG, or with the monoclonal antibody M111 against gp110. Nuclei were visualized with Hoestch (insert). Cultures were viewed with a Nikon Eclipse E800 epifluorescence microscope and photographed with an integrated camera system.

chondroitinase ABC to digest the chondroitin sulfate chains and analyzed by Western blot using a polyclonal antibody against versican raised in our laboratory [10] and with the monoclonal antibody B5 raised against the melanoma-specific proteoglycan mel-CSPG [14]. The results presented in Fig. 1A show that cells with an intermediate differentiation degree (SK-mel-3.44) and all the undifferentiated cell lines (SK-mel-1.36-1–5, SK-mel-37, Rider and AX3) expressed the high molecular weight versican isoforms V0 and V1 in variable amounts, whereas the differentiated cell lines SK-mel-23, MeWo and DX2 cell lines did not express any versican isoform. Mel-CSPG, considered an undifferentiated melanoma marker, was also expressed only in undifferentiated cell lines and absent from the differentiated ones, except the DX2 cells (Fig. 1B). The human astrocytoma cell line U251 was used as a control.

3.2. Detection of versican isoforms in human melanoma cell lines

Only the major V0 and V1 versican isoforms were visualized by Western blot. So, we used RT-PCR and Northern blot assays to analyze the production of V2 and V3 isoforms in our panel of melanoma cell lines. The human astrocytoma cell line U251 was used as a control since it has been described that astrocytomas express all four versican splice variants [16].

RT-PCR results are illustrated in Fig. 2. None of the differentiated melanoma cell lines (SK-mel-23, MeWo and DX-2) expressed any of the four isoforms. All cell lines with an early or intermediate differentiation degree (SK-mel-1.36-1–5, SK-mel-37, Rider and SK-mel-3.44) expressed V0 and V1 transcripts. V2 and V3 expression was only shown by the undifferentiated cell lines SK-mel-1.36-1–5, SK-mel-37 and Rider. However, no quantitative assertion could be made based on these experiments. Therefore, we prepared Northern blots that yielded similar results (Fig. 3). Transcripts for V0 and V1 were visualized in all the undifferentiated and intermediate cell lines using a probe for GAG-β, as a double band around 13 kb and a duplex at 10 kb as has been described for U251 astrocytoma cells [15]. V2 was observed only in the undifferentiated cell lines SK-mel-1.36-1–5 and Rider by using a probe for the GAG-α subdomain requiring a six-times longer exposure period. With a probe prepared for the HABR region common to all the four isoforms, we could only detect two bands corresponding to V0 and V1, indicating that V2 transcript expression is lower. V3 transcript remained below the Northern blot detection limit. Differentiated cell lines did not show any versican isoform. We also checked whether the isoform pattern was altered by the degree of confluence, but this condition did not change the versican isoform expression (not shown).

3.3. Expression of versican along the differentiation process of melanoma cells

The abovementioned results show a close relationship between versican expression and cell differentiation. Therefore, we induced the differentiation process in vitro by treating cultures from SK-mel-1.36-1–5 and SK-mel-3.44 with the differentiation agent TPA [1]. Morphological changes induced by TPA are shown in Fig. 4. This process has been fully characterized by determining the expression of a number of differentiation antigens. Mel-CSPG is one of these antigens since it is expressed only by undifferentiated cells and disappears in TPA-differentiated melanoma cells. Other antigens are induced by the differentiation process, as gp110, gp180 or the melanosome antigen, and are considered late markers. They are recognized by the monoclonal antibodies M111, C350 and CF21, respectively [1]. Therefore,

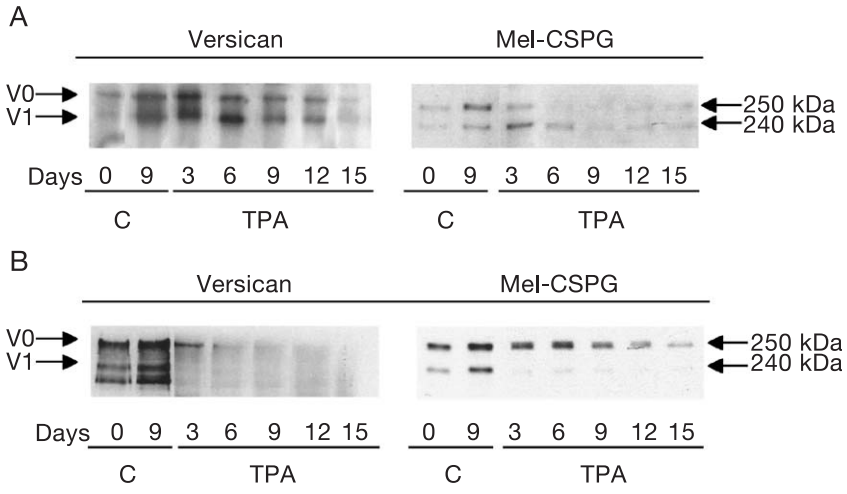

Fig. 6. Versican and mel-CSPG expression during the process of melanoma cell differentiation analyzed by Western blot. Subconfluent SK-mel-3.44 (A) and SK-mel-1.36-1–5 (B) melanoma cells were treated with 160 nM TPA to induce cell differentiation as described in Materials and methods. At the indicated times, conditioned media were collected, digested with chondroitinase ABC and analyzed by Western blot with the anti-versican antibody or the monoclonal antibody B5 against mel-CSPG. The 240 kDa and 250 kDa bands corresponding to distinct glycosylated forms of mel-CSPG are shown.

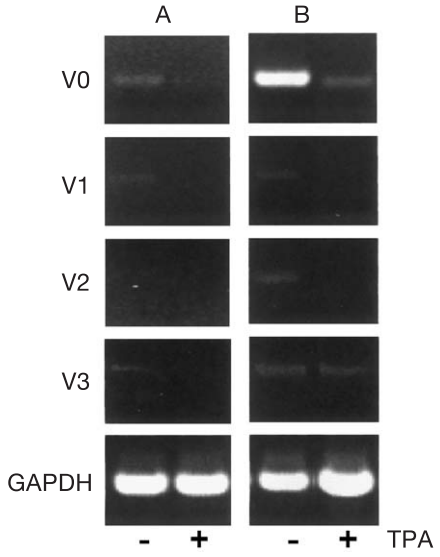

Fig. 7. Expression of versican isoforms during the process of melanoma cell differentiation analyzed by RT-PCR. Subconfluent SK-mel-3.44 (A) and SK-mel-1.36-1–5 (B) melanoma cells were treated with 160 nM TPA to induce cell differentiation as described in Materials and methods. Before treatment and after 15 days in the presence of TPA, total RNA was extracted and RT-PCR analysis was performed as described in Materials and methods.

we monitored the expression of these antigens as markers to follow the differentiation process.

Versican disappears 15 days after induction of SK-mel-1.36-1–5 and SK-mel-3.44 cells differentiation, similarly to mel-CSPG expression, as shown by immunocytochemistry experiments, whereas the gp110 antigen recognized by M111 is induced after TPA treatment (Fig. 5). C350 and CF21 showed a pattern similar to M111 (not shown). A more detailed analysis has been performed by Western blot from cell-conditioned media collected at several days along the differentiation process (Fig. 6). Mel-CSPG rapidly disappears in SK-mel-3.44 cells whereas it decreases at a slower rate in SK-mel-1.36-1–5 cells. Versican expression does not follow the same pattern since its disappearance rate is slower in SK-mel-3.44 cells. Both V0 and V1 isoforms decrease in a parallel way.

We subsequently looked at the four versican isoforms expression by RT-PCR in SK-mel-1.36-1–5 and SK-mel-3.44 cells before and after TPA-induced differentiation (Fig. 7). Control SK-mel-3.44 cells expressed V0, V1 and V3 isoforms, whereas we could detect only V0 in induced cells. In SK-mel-1.36-1–5 cells, we could detect V0 and V3 after differentiation although V1 and V2 were undetectable.

4. Discussion

It is widely accepted that during tumor progression, in which cells acquire their enhanced ability to proliferate and migrate, cells regress to an undifferentiated phenotype. In melanoma, this process is characterized by the expression or repression at different periods of a wide range of molecules and, in consequence, the panel of antigens expressed by a selected melanoma or by melanoma cell lines defines their differentiation degree [2]. It has been proposed that the three differentiation degrees (early, intermediate and late) of melanoma tumor cell lines would correspond to the normal melanocyte features at the early, intermediate or mature phases in melanocyte differentiation, as they express a similar set of differentiation antigens.

In a previous work, we have identified the expression of a new malignant melanoma marker, the extracellular proteoglycan versican. Versican is expressed in a high percentage of malignant melanomas [10], whereas the expression is much lower in dysplastic nevi and null in benign melanocytic nevi [17]. This finding supports the existence of a melanoma progression pathway, where the dysplastic nevus represents a precursor of malignant melanoma [18]. In the present work, we have analyzed the expression of different versican isoforms in melanoma cell lines, to study the relationship between the expression of this proteoglycan and the degree of cell differentiation in vitro, which would correspond to different stages in melanoma development. Our results show that, extending our previous findings, SK-mel-23, MeWo and DX2 differentiated melanoma cell lines do not produce any versican isoform. The early-differentiated cells (SK-mel-1.36-1–5, SK-mel-37 and Rider) produce all versican isoforms, as detected by RT-PCR, although quantitative differences were detected when using Northern blot analysis. In this case, V0 and V1 were the most abundant isoforms in undifferentiated cell lines. V0 isoform, which has been suggested as the prevalent one during early embryonic development [19–21] is present here. The V2 isoform, which is supposed to be abundant in neural tissues [22], is also present, perhaps as a consequence of their neuroectodermal origin. Nevertheless, the level of V2 should be much lower than V0/V1 isoforms, since the time of exposure for the Northern blot had to be six times longer to get similar band intensity. Furthermore, we could not detect any band corresponding to V2 in Western blot, although our antibody is able to recognize a band similar to V2 in bovine brain extracts (not shown). The short V3 isoform is detected by RT-PCR, although we could not detect it by Northern blot. SK-mel-3.44 cells, that have an intermediate degree of differentiation, show the presence of V0, V1 and V3 by Northern blot and RT-PCR. In consequence, we propose that the loss of the differentiated state in melanoma cells is accompanied by the expression of versican isoforms. From the differences between cell lines in an intermediate or early differentiation degree, we suggest that V0, V1 and V3 are expressed first, and finally V2.

These differences may have functional consequences: although all isoforms share the same N- and C-terminal domains and should be able to establish the same interactions with other molecules, the central domain is differently spliced. Therefore, the number of glycosaminoglycan chains

is also different: whereas the V0 isoform carries the highest amount of GAG chains and therefore is the most negatively charged, V1 and V2 have a minor amount of chains and a minor charge, and V3 has no GAG chains. The existing distance between the N- and C-terminal domains should also be different. These differences should affect the ability of versican to interact with its ligands and, most probably, its physiological properties. Although there are no definitive studies on the possible differential biological role of the isoforms, it has been suggested that GAG domain size could modulate versican axonal growth inhibitory capacity in the nervous system [23].

To ascertain if this process is reversible, we assessed whether versican expression is down regulated in undifferentiated SK-mel-3.44 and SK-mel-1.36-1–5 melanoma cells grown in a differentiation-promoting conditions, i.e., in presence of TPA. The differentiation process has been fully characterized in these specific cell lines [1], and it is widely accepted that TPA suppresses the growth of melanoma cells, and that this process is frequently accompanied by terminal differentiation [24–26]. Our results demonstrate that versican expression decreases in these conditions, linking differentiation state and versican expression. In both cases, we used mel-CSPG expression, a well-known melanoma-associated antigen present in undifferentiated cell lines and absent from differentiated ones, and gp110, a late differentiation antigen for melanoma, as markers to follow the process. The expression of V0 and V1, the two isoforms detected by Western blot, decreases at a parallel rate, although V0 remains detectable by RT-PCR, as well as V3 in SK-mel-1.36–5 cells. There are other indications in the literature that relate versican expression and cell proliferation/differentiation, although there have been no studies on the presence of particular isoforms. Thus, versican is down-regulated in culture conditions promoting keratinocyte differentiation but is up-regulated in keratinocytes grown in proliferation-promoting conditions [27].

Although the molecular mechanism for versican regulation during cell differentiation is not known, it is clearly established that versican isoform expression and splicing are developmentally regulated, especially in the nervous system and during neural crest migration and differentiation [20,21,28]. Recently, it has been also described that the versican gene may be a target for Pax3, a transcription factor involved in neural crest cell development. Pax3 is expressed in a spatiotemporally restricted manner during embryogenesis [29], and it has been suggested that it regulates the expression of genes involved in cell adhesion and mobility, influencing in this manner the migration of neural crest cells, which will give origin to melanocytes [30]. Pax3 is overexpressed in malignant melanoma and not in benign nevi [31]. It has been recently described that versican V2 isoform is up-regulated in Pax3-transfected cells, although no consensus sequence for Pax3 is found in the versican promoter [32]. Nevertheless, the versican promoter includes several other regulatory sites (AP2, SP1, CTF/CBF, CBP, CREB and C/EBP) [33], and some of these transcription factors are developmentally regulated [34,35].

Finally, it is interesting to note that all the undifferentiated cell lines produce the short V3 isoform. This isoform lacks GAG chains, and it has been described that it may have opposite functional roles to the large isoforms by exerting a functional competition with them [36]. The expression of V3 has been reported in several cells and tissues as adult brain, fetal liver [37,38], smooth muscle cells [39], and others [19]. In endothelial cells, V3 is specifically expressed after activation with cytokines as TNF-α or VEGF, being therefore associated with neoangiogenesis and wound healing processes [19]. In our case, V3 remains detectable after the induction of melanoma cell differentiation, raising the possibility of a specific role for this isoform.

In conclusion, we propose that versican production by melanoma cells is a sign of regression to an undifferentiated state during tumor progression, and that there is a different time appearance pattern for versican isoforms.

Acknowledgements

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